Regulation of Invasiveness of Glioblastoma Multiforme Cell Lines and Tumor Stem Cells by Sphingosine Kinase and Sphingosine 1-Phosphate Reveals Potential Molecular Therapeutic Targets

Nicholas Young*

Abstract

Glioblastoma Multiforme (GBM), the most common primary brain tumor in adult patients, is a grade IV astrocytic neoplasm characterized histologically by high mitotic index, significant angiogenesis, necrosis, and local invasion. This infiltrative nature leads to inevitable tumor recurrence even after conventional radiation and chemotherapeutic treatment. With a median patient survival of less than one year, there is a great need for a better understanding of the molecular basis of these malignant behaviors in order to develop effective molecular based therapies.

We have been investigating the role played by the bioactive lipid sphingosine-1-phosphate (S1P) in GBM. S1P stimulates growth, motility, invasiveness, angiogenesis, and adhesion in a variety of tumor cell types. We have previously shown that S1P stimulates growth and invasion of glioma cells through its receptors ($S1P_{1-3}$) which are commonly expressed in GBM tissue. In addition, we have also shown that high levels of the enzyme responsible for S1P production, sphingosine kinase (SphK), correlate with *poorer survival of GBM patients. This study uses receptor manipulated GBM cell lines to delineate the contributions of the various S1P receptors to the malignant behavior of well established GBM cell lines. The results show that $S1P_1$ and $S1P_3$ stimulate growth, motility, and invasion. $S1P_2$ also stimulates GBM cell growth.

* Integrated Biomedical Science Program, The Ohio State University, Columbus OH, 43210, E-mail: young.651@osu.edu, I would like to thank Dr. James Van Brocklyn for his insightful comments on earlier drafts.

Although $S1P_2$ decreases GBM cell motility, surprisingly it increases invasion, by enhancing attachment to extracellular matrix material. This unusual enhancement of invasion by $S1P_2$ also required upregulation of the matricellular protein CCN1/Cyr61, since this effect was blocked by neutralizing antibodies against this effector molecule.

Furthermore, we have begun to investigate the role of S1P in both normal neural stem cells and brain tumor stem cells (BTSC) derived from GBM tissue. BTSC are a subpopulation of cells present in GBM which, unlike traditional GBM cell lines, form tumors in animal models that resemble GBMs histologically and invade into surrounding brain tissue. BTSC are likely the formative cells of GBM tumors and may be derived from the transformation of normal neural stem cells. Spheroid invasion assays show that S1P stimulates invasion of both BTSC and neural stem cells, while SphK inhibitors block the invasion of BTSC. These data suggest that these cells are highly responsive to S1P and that enhanced SphK in GBM BTSC could drive the invasion of these cells. In summary, our data identify downstream mediators of S1P in GBM cells that may mediate enhanced malignant behavior of these tumors. Several of these mediators may be useful as therapeutic targets. In addition, our data implicate S1P and SphK in the invasiveness of the crucial BTSC.

Introduction

Sphingosine-1-phosphate (S1P) is a bioactive lipid that regulates cellular proliferation, migration, survival, cytoskeletal rearrangement, and angiogenesis (Spiegel and Milstien 2003; Hla 2004; Young and Van Brocklyn 2006). With the discovery of the S1P family of receptors throughout the 1990's, autocrine/paracrine receptor stimulation influence on cellular activity has been under intense investigation. S1P signals both intracellularly as a second messenger

(Spiegel and Milstien 2003) and through five cell surface, G protein-coupled receptors (GPCRs) $S1P_{1-5}$ (Young and Van Brocklyn 2006). Knock out models in mice have shown the critical individual receptor subtype importance in establishing a normal phenotype (Ishii, Ye et al. 2002). Before identification of receptors, sphingosine kinase (SK) production of S1P was thought to solely be responsible for modulating the intracellular effects of this lipid. Presently, the specific signaling pathways of both intracellular S1P as well as extracellular receptor stimulation remain to be conclusively elucidated.

Our group has been exploring the roles played by S1P in the growth and invasiveness of glioblastoma multiforme (GBM) cells. GBM is the most commonly occurring primary brain tumor in adults and is highly malignant, displaying aggressive growth and invasion into surrounding brain tissue (Lefranc, Sadeghi et al. 2006) leading to a median life expectancy of only 10-12 months following diagnosis (Sathornsumetee and Rich 2006). A better understanding of the molecular regulation of GBM cell growth and invasion will be necessary to develop effective molecular-based therapies.

Upon S1P receptor stimulation, G-protein pathways lead to distinct cellular effects that have been observed and characterized both *in vitro* and *in vivo*. We have shown that S1P is mitogenic for (Van Brocklyn, Letterle et al. 2002), and enhances motility and invasiveness of GBM cell lines (Van Brocklyn, Young et al. 2003). As an indication of the importance of this signaling system for *in vivo* GBM, high expression levels of the enzyme which forms S1P, sphingosine kinase-1 (SphK1), in GBM tissue correlate with a more than 3 fold shorter survival time of GBM patients (Figure 1) (Van Brocklyn, Jackson et al. 2005). Furthermore, knockdown of SphK1 or SphK2 expression using RNA interference decreases GBM cell proliferation by preventing entry of cells in the cell cycle (Van Brocklyn, Jackson et al. 2005).

The mitogenic and invasive effects of S1P on GBM cells are at least partially mediated through its GPCRs, since both responses are sensitive to pertussis toxin (Van Brocklyn, Letterle et al. 2002), which specifically inhibits signaling through the $G_{i/o}$ family of G proteins. Moreover, S1P induction of both mitogenesis and invasiveness of GBM cells occurs at nanomolar concentrations, consistent with the affinities of S1P for its receptors (Van Brocklyn, Letterle et al. 2002). GBM cell lines (Van Brocklyn, Young et al. 2003) and GBM tissue (Van Brocklyn, Jackson et al. 2005) commonly express three S1P receptors, $S1P_1$, $S1P_2$ and $S1P_3$. $S1P_4$, which is primarily expressed in cells of hematopoietic origin (Gräler, Bernhardt et al. 1998), has not been detected in gliomas. $S1P_5$ is expressed in normal brain in oligodendrocytes (Jaillard, Harrison et al. 2005), however we have detected only very low levels of $S1P_5$ expression in a limited number of GBM cases and several glioma cell lines (unpublished observation). Unfortunately, a suitable *in vivo* model of GBM progression employing traditional cell lines is not available. Here, we establish a relevant model to use in nude mice through the use of the GBM cell line X-12, which is passaged in the flanks of nude mice rather than in culture using traditional *in vitro* methods. This cell line is thought contain an enriched population of transformed neural stem cells that serve as the formative brain tumor stem cells (BTSC) in GBM tumors. We were able to histopathologically recapitulate the local invasiveness characteristically observed in human GBM cases through intracranial injections into nude mice (Figure 2).

Each S1P receptor subtype activates a unique set of G proteins with varying preferences (Taha, Argraves et al. 2004; Young and Van Brocklyn 2006). Therefore, the individual influence of each receptor subtype on S1P regulation of GBM cell behavior may depend on the amounts of the individual S1P receptors expressed.

Results and Discussion

In this study, the effects of S1P through individual receptor subtypes on glioma cell growth, migration, invasion, and adhesion were examined by overexpressing individual S1P receptors in glioma cells with low endogenous receptor levels, and knocking down individual S1P receptor expression by RNA interference in glioma cells with high levels of expression (Table 1). The results show that $S1P_1$, $S1P_2$ and $S1P_3$ all contribute to glioma cell growth (Figure 3) and invasion (Figure 4) through distinct, but overlapping, mechanisms. Conversely, $S1P_5$ inhibits these cellular activities when overexpressed in glioma cells (Figures 3-5). Furthermore, the $S1P_2$ receptor subtype displays a novel enhancement of S1P-stimulated invasion while decreasing migration (Figure 4). The $S1P_2$-stimulated invasiveness in these glioma cell lines correlates with enhanced cell adhesion (Figure 5) and is mediated, at least partially, by the matricellular protein CCN1/Cyr61 (Figure 6). Additionally, the $S1P_2$ mediated response was also shown in a spheroid invasion assay (Figure 7), which serves as a more relevant model to gauge *in vivo* tumor invasiveness (Bauman, Fisher et al. 1999). This differential influence of the same ligand is therefore dependent on the receptor subtype expressed by the cell. Since each S1P receptor subtype signals through a unique set of G-proteins that result in various downstream cellular effects, it is then critical to know what receptors are present in order to predict cellular behavior with ligand stimulation.

The X-12 GBM cell line was then examined to identify S1P induced effects on invasiveness. Since these cells are thought to potentially arise from neural stem cell transformation, we also used the neural stem cell line SFME to compare in a spheroid invasion assay. S1P clearly induced invasiveness in neural stem cells and we interestingly found that treatment with S1P promoted additional extracellular matrix infiltration above that observed at

endogenous levels in X-12 GBM cells (Figure 8). Furthermore, Sphk inhibitor effectively reduced this endogenous response (Figure 9). We therefore conclude that the X-12 GBM cell line resembles neural stem cells in S1P induced invasiveness and that Sphk expression plays a key role in the endogenous X-12 invasion response.

With such dramatic cellular effects influencing these GPCRs individually with S1P presence, understanding the role of activation of each subtype can have great medical impact. In 2001, 50% of current commercially available pharmaceuticals targeted GPCRs and were a $30 billion dollar industry (Klabunde and Hessler 2002). In addition, these pharmaceuticals represented approximately one quarter of the top 100 selling drugs. Furthermore, biological influence of these mechanisms is apparent considering that S1P receptors are present in virtually every tissue and cell type and S1P blood levels range from 0.1-1μM, which is greater than the K_d of each receptor (Goetzl and Rosen 2004). Also, SphK1 overexpression has been correlated to several other forms of cancer, including breast, lung, and colon tumors when compared to normal tissue (French, Schrecengost et al. 2003). Although some functional redundancy is evident, the extent of preferential G protein pathway activation has yet to be examined and characterized in detail for each individual receptor subtype. With this understanding, opportune clinical application in the molecular based therapeutic treatment of a variety of cancers will be possible.

Table 1: S1P receptor expression levels in stably-transfected U-118 MG and U-373 MG cell lines

		Percent expression relative to control cell lines			
Cell line	**transfection**	**$S1P_1$**	**$S1P_2$**	**$S1P_3$**	**$S1P_5$**
U-118-$S1P_1$A	$S1P_1$	**682**	87	106	116
U-118-$S1P_1$B	$S1P_1$	**408**	ND	ND	ND
U-118-$S1P_2$A	$S1P_2$	90	**200**	97	110
U-118-$S1P_2$B	$S1P_2$	ND	**459**	ND	ND
U-118-$S1P_3$A	$S1P_3$	90	97	**428**	112
U-118-$S1P_5$A	$S1P_5$	119	115	88	**216**
U-118-$S1P_5$B	$S1P_5$	ND	ND	ND	**444**
U-373-pSil-1A	si$S1P_1$	**8**	118	98	116
U-373-pSil-1B	si$S1P_1$	**30**	ND	ND	ND
U-373-pSil-2A	si$S1P_2$	92	**33**	118	110
U-373-pSil-2B	si$S1P_2$	ND	**36**	ND	ND
U-373-pSil-3A	si$S1P_3$	104	120	**17**	114
U-373-pSil-3B	si$S1P_3$	ND	ND	**39**	ND

ND = not determined

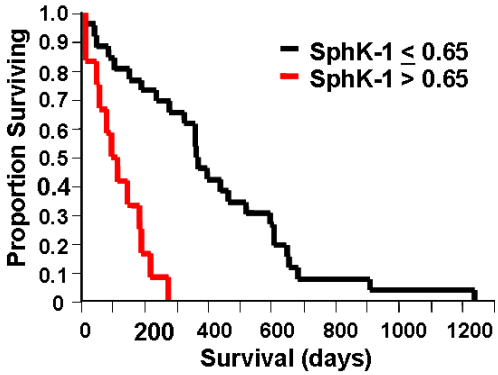


Figure 1. Survival analysis of glioblastoma patients. Tumors were divided into low (≤ 0.65) vs. high (>0.65) sphingosine kinase (SphK)-1 expression level, as determined by quantitative real time PCR analysis. The median survival for the glioblastoma group with SphK1 > 0.65 was 102 days. The median survival for the glioblastoma group with SphK1 ≤ 0.65 was 357 days. The difference is statistically significant, p = 0.002 (n = 48) after correcting for patient age.

X-12 **U-87 MG**

Figure 2. X12 GBM cells, passaged as flank tumors in nude mice, or U-87 MG glioma cells were injected intracranially into nude mice. Upon death of the mice sections of tumor were immunohistochemically stained with an antibody specific for human vimentin. Note cells diffusely invading from X12-derived tumors, in comparison to well circumscribed U-87 MG-derived tumors.

Figure 3. Analysis of growth over time in U-118 MG and U373-MG cell lines. U-373 MG cell lines with siRNA directed against S1P1, S1P2, and S1P3 were measured along with U-118 MG cell lines overexpressing individual receptor subtypes. Cells were plated at a starting concentration of 100,000 cells per well in a 12 well plate and grown in EMEM with 10% FBS for 24 hours and then starved for 24 hours. The wells were then treated with 100nm S1P and allowed to grow. At each indicated time point, cells were trypsinized and counted.

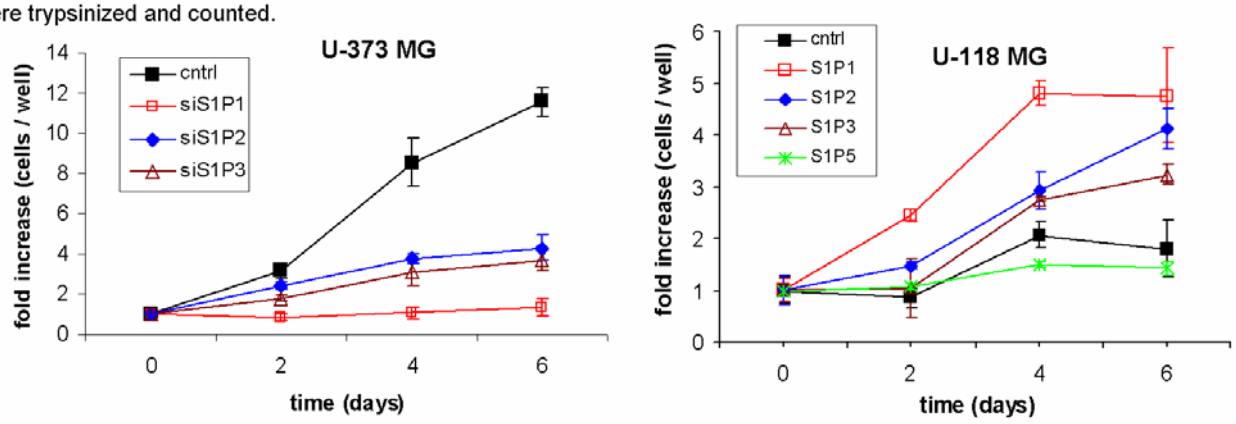
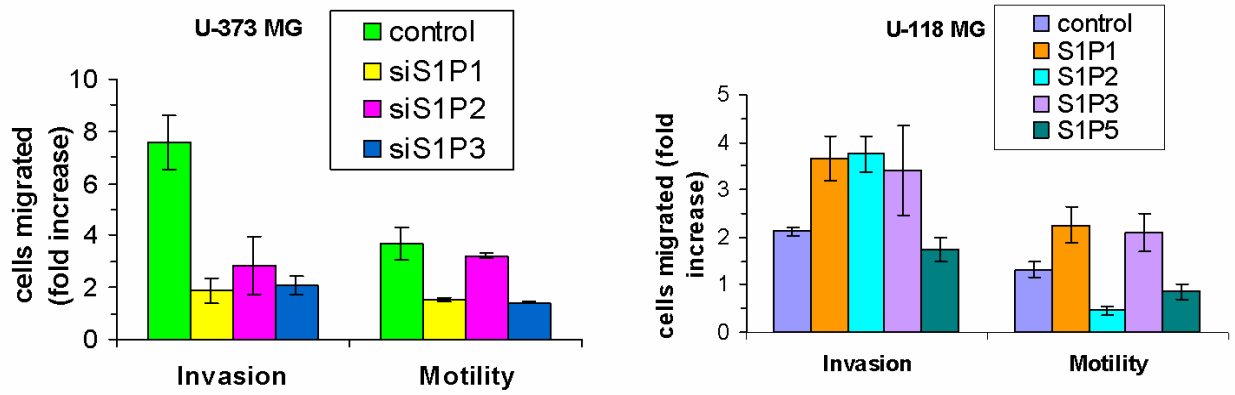


Figure 4. Cellular migration and invasion assay. siRNA receptor-targeted U-373 MG cell lines (A) and U-118 MG cell lines overexpressing S1P2, S1P3, and S1P5 (B) and were plated in Transwell insert 24-well plates coated with a small gelatin dilution (migration) or Matrigel (invasion). Cells were plated at a starting concentration of 100,000 per well. The wells were treated both with and without 100 nM S1P. After a 72 hr incubation, the inserts were removed, cells were stained and quantitated as absorbance at 635nm. Data shown as an increase in S1P mediated response over untreated sample.



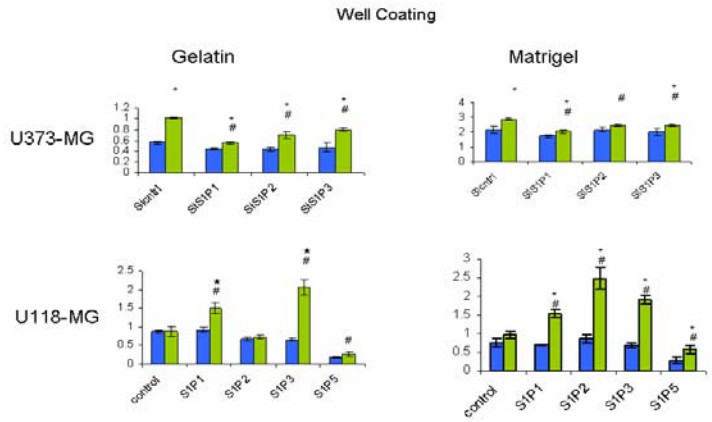


Figure 5. Adhesion assay for U-118 MG and U-373 MG cell lines. Each cell line was grown both with and without S1P presence after an overnight starvation. siRNA receptor-targeted U-373 MG cell lines and U-118 MG cell lines overexpressing S1P2, S1P3, and S1P5 were plated at a concentration of 50,000 cells in 96-well plates coated with a small gelatin dilution or Matrigel. After a 3 hour incubation period, the media was removed and cells were fixed, stained, and quantitated as an absorbance at 540nm. Blue bars indicate 0nm S1P and Green bars indicate 100nm S1P treatment. * represents a statistically significant difference ($p < 0.05$) between treated and untreated samples and # represents a statistically significant difference ($p < 0.05$) between the treated control and the treated cell line.

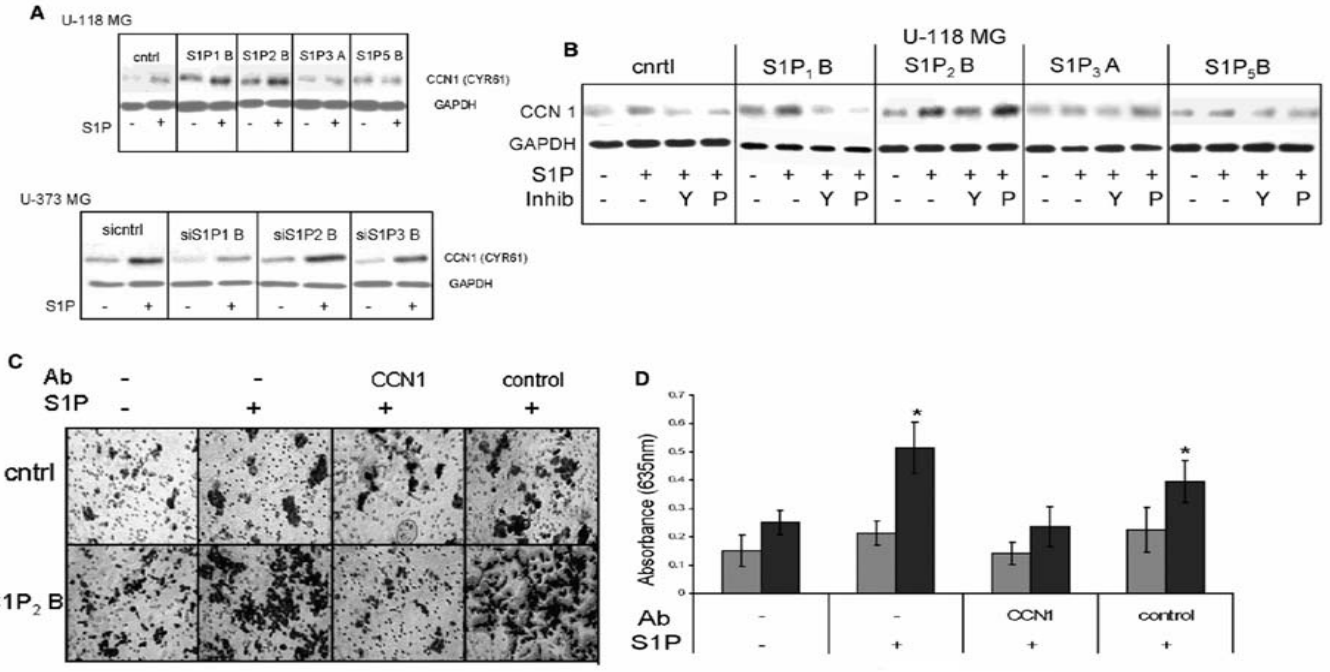


Figure 6. **Role of CCN1/Cyr61 in $S1P_2$-stimulated glioma cell invasion. (A) U-373 MG and U-118 MG cells were stimulated with or without S1P for 3 h. (B) Cells were pretreated with or without 10 μM Y-27632 for 30 min (Y) or with 200 ng/ml PTX (P) for 3 h and then treated with or without 100 nM S1P for 3 h. For both panels A and B cell lysates were subjected to Western blot analysis for CCN1. Blots were stripped of antibodies and reprobed for GAPDH as a loading control. (C) S1P-stimulated invasion of U-118 MG control and $S1P_2$-overexpressing cells was analyzed using the transwell assay in the absence and presence of antibodies to CCN1 or an irrelevant control antibody (anti-pAKT). (D) Results from the experiment shown in panel C were quantitated as described in Materials and methods. Gray bars represent U-118 MG control cells. Black bars represent $S1P_2$-overexpressing cells. The * indicates a statistically significant difference ($p<0.05$) between S1P-treated and untreated cells of the same cell line.**

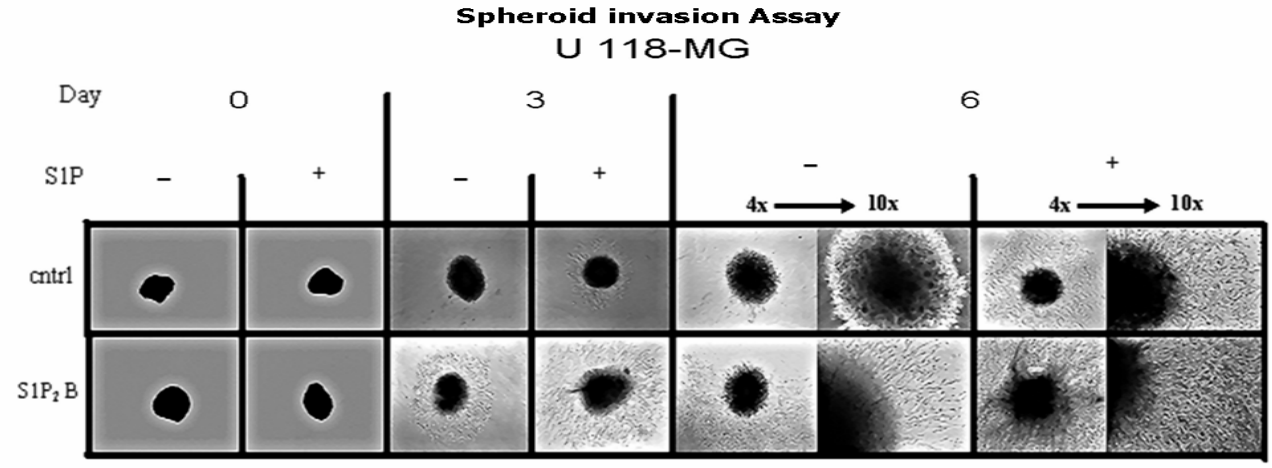


Figure 7. U 118–MG control or $S1P_2$ overexpressing cells were grown inverted for 3 days at a starting concentration of 50,000 cells. The resulting spheres were transplanted into a Matrigel matrix without and with 100nm S1P in serum free media. Spheroid invasion was observed through 4x and 10x photographs taken at the indicated time points.

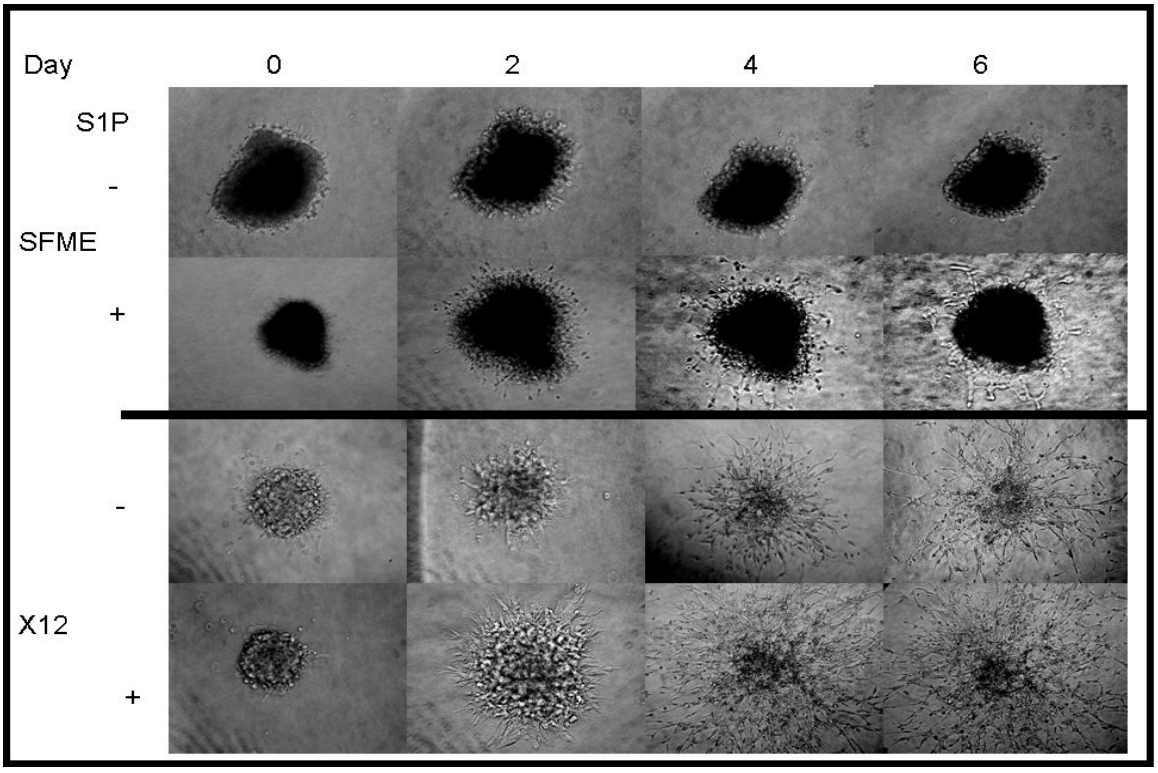


Figure 8. SFME or X-12 cells were grown as neurospheres in defined media suspension before being transplanted into a Matrigel matrix without and with 100nm S1P in serum free media. Spheroid invasion was observed through 10x photographs taken at the indicated time points.

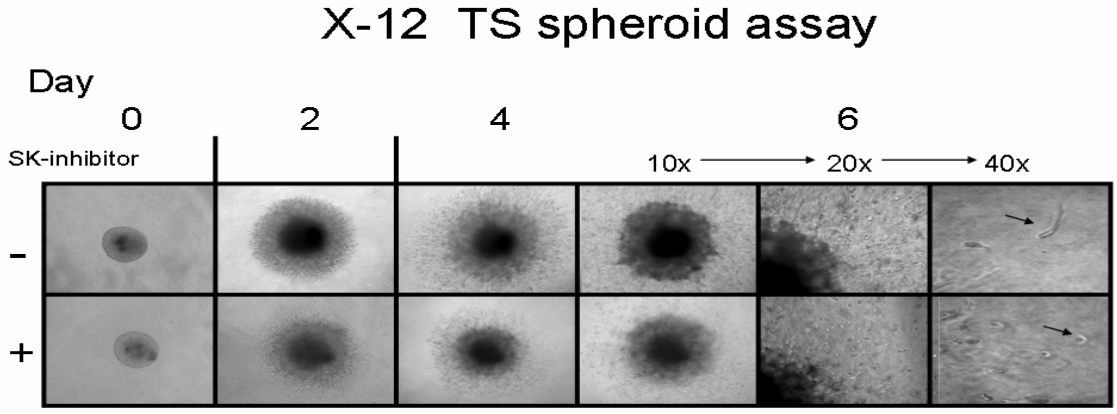


Figure 9. SFME or X-12 cells were grown as neurospheres in defined media suspension before being transplanted into a Matrigel matrix without and with sphingosine kinase inhibitor in serum free media. Spheroid invasion was observed through 10x, 20x, and 40x photographs taken at the indicated time points.

References:

Bauman, G. S., B. J. Fisher, et al. (1999). "Effects of radiation on a three-dimensional model of malignant glioma invasion." Int. J. Dev. Neurosci. **17**(5-6): 643-651.

French, K. J., R. S. Schrecengost, et al. (2003). "Discovery and evaluation of inhibitors of human sphingosine kinase." Cancer Res. **63**(18): 5962-2969.

Goetzl, E. J. and H. Rosen (2004). "Regulation of immunity by lysosphingolipids and their G protein-coupled receptors." J. Clin. Invest. **114**(11): 1531-1537.

Gräler, M. H., G. Bernhardt, et al. (1998). "EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue." Genomics **53**(2): 164-169.

Hla, T. (2004). "Physiological and pathological actions of sphingosine 1-phosphate." Semin. Cell Dev. Biol. **15**(5): 513-520.

Ishii, I., X. Ye, et al. (2002). "Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate receptors, S1P2/LPB2/EDG-5 and S1P3/LPB3/EDG-3." J. Biol. Chem. **277**(28): 25152-25159.

Jaillard, C., S. Harrison, et al. (2005). "Edg8/S1P5: an oligodendroglial receptor with dual function on process retraction and cell survival." J. Neurosci. **25**(6): 1459-1469.

Klabunde, T. and G. Hessler (2002). "Drug design strategies for targeting G-protein-coupled receptors." Chembiochem **3**(10): 928-944.

Lefranc, F., N. Sadeghi, et al. (2006). "Present and potential future issues in glioblastoma treatment." Expert Rev Anticancer Ther **6**(5): 719-32.

Sathornsumetee, S. and J. N. Rich (2006). "New treatment strategies for malignant gliomas." Expert Rev Anticancer Ther **6**(7): 1087-104.

Spiegel, S. and S. Milstien (2003). "Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways." Biochem. Soc. Trans. **31**(Pt 6): 1216-1219.

Spiegel, S. and S. Milstien (2003). "Sphingosine-1-phosphate: an enigmatic signalling lipid." Nat. Rev. Mol. Cell Biol. **4**(5): 397-407.

Taha, T. A., K. M. Argraves, et al. (2004). "Sphingosine-1-phosphate receptors: receptor specificity versus functional redundancy." Biochim. Biophys. Acta **1682**(1-3): 48-55.

Van Brocklyn, J. R., C. A. Jackson, et al. (2005). "Sphingosine kinase-1 expression correlates with poor survival of patients with glioblastoma multiforme. Roles of sphingosine kinase isoforms in growth of glioblastoma cell lines." J. Neuropathol. Exp. Neurol. **64**(8): 695-705.

Van Brocklyn, J. R., C. A. Letterle, et al. (2002). "Sphingosine-1-phosphate stimulates human glioma cell proliferation through G_i-coupled receptors: Role of ERK MAP kinase and phosphatidylinositol 3-kinase β." Cancer Lett. **181**(2): 195-204.

Van Brocklyn, J. R., N. Young, et al. (2003). "Sphingosine-1-phosphate stimulates motility and invasiveness of human glioblastoma multiforme cells." Cancer Lett. **199**(1): 53-60.

Young, N. and J. R. Van Brocklyn (2006). "Signal transduction of sphingosine-1-phosphate G protein-coupled receptors." ScientificWorldJournal **6**: 946-66.